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Bioensaios ecotoxicológicos para avaliação de efeitos de substâncias químicas em solo

Ecotoxicological bioassays to evaluate the effects of chemicals in soil

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Terrestrial ecotoxicological bioassays to evaluate the effects of chemicals for environmental risk assessment

Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Toxicologia e Ecotoxicologia, realizada sob a orientação científica do Professor Doutor Amadeu Soares, Professor catedrático do Departamento de Biologia da Universidade de Aveiro e co-orientação da Doutora Mónica Amorim, Investigadora Auxiliar do CESAM, Departamento de Biologia da Universidade de Aveiro.

o júri

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palavras-chave

Produtos de higiene pessoal, Compostos policíclicos aromáticos, Toxicologia terrestre e ecotoxicologia.

resumo

A utilização de compostos químicos e os consequentes efeitos em distintos compartimentos ambientais tem despertado muito interesse nos últimos anos. O aparecimento de Produtos de Higiene Pessoal no ambiente tem sido considerado como uma questão a ser colocada no domínio ambiental. A sua presença no ambiente ocorre devido á excessiva utilização por parte dos consumidores e à sua incompleta remoção das estações de tratamento de águas residuais. Nessas estações os efluentes tratados e lamas são novamente reintroduzidos no ambiente e na maioria dos casos afectam os organismos residentes nas comunidades locais. Um outro tipo de compostos que também têm recebido atenção especial é os Hidrocarbonetos Policíclicos Aromáticos (HPAs), que desde a sua primeira aparição no solo, despertaram grande curiosidade. Estes compostos são derivados da combustão incompleta de compostos contendo carbono e hidrogénio e do processo diagénese.

Com este trabalho pretendemos avaliar a toxicidade do Triclosan num conjunto de organismos-teste terrestres, lumbricideos (*Eisenia andrei*), colêmbolos (*Folsomia candida*), enquitraideos (*Enchytraeus albidus*), incluindo dois tipos de plantas superiores (*Triticum aestivum* e *Brassica rapa*). Os parâmetros avaliados foram a germinação e crescimento das plantas e a sobrevivência /reprodução dos restantes organismos. Também foi realizado um ensaio de bioacumulação para avaliar o comportamento cinético de um HPA, o fenantreno em *E. albidus*. Para a determinação da concentração de fenantreno nos organismos bem como nos extractos de solo dois métodos foram usados: um por fluorescência e outro por cromatografia gasosa com espectometria de massa acoplado.

Os resultados obtidos para a bateria de testes mostram uma relação dose-resposta para todas as espécies estudadas, *Eisenia andrei* é a que demonstra ser mais sensível, com um valor de CE_{50} de aproximadamente 4mg/kg; a sensibilidade para cada espécie por ordem decrescente é a seguinte *E. andrei* > *B. rapa*> *E. albidus* > *F. candida* > *T. aestivum*. Verificou-se que os enquitraideos podem acumular e ser afectados por uma variedade de compostos orgânicos e inorgânicos, tais como os HPAs (fenantreno). Os parâmetros cinéticos estimados para o fenantreno foram 4.4 g solo/g organismo dia^{-1} para a taxa de assimilação (a) e 0,305 dia-1 para a taxa de eliminação (k_e), o factor de bioacumulação calculado é de 14.3 para *E. albidus.* Todos os ensaios foram realizados com um solo padronizado Lufa 2.2.

No âmbito geral, os testes ecotoxicológicos realizados, revelam a importância de incluir diversas espécies, abrangendo diferentes níveis tróficos, devido á distinta sensibilidade dos organismos e modos de acção dos diferentes químicos.

keywords Personal Care Products, Polycyclic Aromatic Compounds, soil toxicology, Ecotoxicology

abstract

The usage of chemical compounds and their effects in the different environmental compartments have received a special attention in recent years. The occurrence of Personal Care Products (PCPs) in the environment has been recognised as one of emerging issues in environmental chemistry. The widespread presence of PCPs in the environment is due to their extensive use for direct use by the consumer and incomplete removal in wastewater treatment plants (WWTPs). WWTPs produce aqueous effluents for discharge back into the environment, and sewage sludge and in a number of cases may affect the living organisms. Although Polycyclic Aromatic Compounds (PAHs) have received special attention since they were found in soils for the first time. PAHs are a class of several hundreds individual compounds containing at least two condensed rings. They are produced by the incomplete combustion of compounds containing C and H, and diagenesis.

With this study we assessed the toxicity of Triclosan in several standardized test organisms and parameters: seed emergence and growth of two terrestrial plants (*Triticum aestivum* and *Brassica rapa*); survival and reproduction of earthworms (*Eisenia andrei*), collembolans (*Folsomia candida*) and enchytraeids (*Enchytraeus albidus*). The results for the test battery shows a dose-response relationship for the all organisms tested and *Eisenia andrei* was the most sensitive specie, with an EC_{50} value of approximately 4mg/kg; with species chronic sensitivity decreasing from *E. andrei* > *B. rapa* > *E. albidus* > *F. candida* > *T. aestivum*. The overall results from the selected ecotoxicological tests, showed the importance of including species from different trophic levels due to the different species sensitivities and chemicals mode of action. Moreover, also the use of chronic endpoints is recommended.In the case of the PAH phenantrene (PHE), a study was performed in *E. albidus*, assessing the effects at the survival and reproduction, plus the bioaccumulation, to analyse the toxicokinetic behaviour of this chemical. *Enchytraeus* can accumulate and may be affected by PHE. Estimated kinetic parameters were 4.4g soil/g worm day^{-1} for the assimilation rate (a) and 0,305 day^{-1} for the elimination rate constant (k_e), bioaccumulation factor was 14.3. In a general trend, the ecotoxicological tests performed show the importance, of study different trophic levels, including different species, due to the different sensibility of the organisms and different chemicals modes of action.

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CHAPTER I

Ecotoxicology, thesis structure and objectives

Introduction – Ecotoxicology

The term Ecotoxicology was first introduced in 1969 by Professor R. Truhaut, who defined it as "the study of adverse effects of chemicals with the aim of protecting natural species and populations" (Twardowska, 2004). Truhaut's definition of ecotoxicology has been followed by several others. Ecotoxicology is a multidisciplinary science, combining the fields of chemistry, toxicology, pharmacology, epidemiology and ecology with an understanding of the sources and fates of chemicals in the environment. The major concerns are the interactions between the toxic compounds and the living organisms, from the molecular to the population and biosphere (e.g., global warming) levels of biological organization. Therefore, the main tasks of Ecotoxicology are to assess, monitor and predict the fate and effects of foreign substances in the environment (Moriarty, 1988).

To evaluate and predict the effects of chemical substances on the terrestrial environment, standardized soil ecotoxicology tests have been developed. As a result, these toxicity tests are used for three main purposes: (a) prediction of the effects of chemical substances in the soil compartment; (b) comparison of sensitivity of one or more species to different toxicants, or to different test conditions for the same toxicant; (c) setting rules for regulation of the compounds usage (Baudo, 1987).

To fulfil these purposes, and for an adequate risk assessment, it is crucial an appropriate selection of the test species. Test organisms should be representative of different taxonomical or physiological groups, have different ecological function and life-history strategies, should belong to different trophic levels and should have different routes of exposure, among other characteristics (Laskowsky *et al.*, 1998). An example of suitable test organisms are earthworms, important members of the soil community due to their activity, being able to improve soil structure, stabilize soil aggregates, increase water infiltration and water-holding capacity and form a humus layer close to the surface. Several studies have been performed with *Eisenia andrei* and *Eisenia fetida*. Collembolans, other test organisms, are the most numerous and widely distributed terrestrial insects. They have an important role as regulators of decomposition processes through microbivory and microfauna predation. *Folsomia candida* (Collembola) is the most used species of Collembola in ecotoxicological research. Another example of suitable test organisms are enchytraeids. They belong to the saprophagous mesofauna of the litter layer and the upper mineral soil and contribute to vital processes of this environmental compartment. Indirectly

they are involved in regulating the degradation of organic matter, as well as improving the pore structure of the soil (Amorim et al., 2005). *Enchytraeus albidus* is the best-known and one of the largest species of the genus *Enchytraeus* and it has been increasingly used for ecotoxicology purposes. The plants *Brassica rapa* and *Triticum aestivum* are two standard species largely used for the assessment of growth and germination. They are primary producers and play an important role to the regulation of the environment.

A complete ecotoxicological assessment of the soil ecosystem requires a good battery of test organisms, like the ones previously mentioned, but also different endpoints in order to establish a relationship between the concentration of chemicals in the environment, and the responses they elicit in living organisms (including survival, reproduction and bioaccumulation) (Römbke, 2003).

Ecotoxixology tests that assess survival are primarily to examine the short-term (acute) effects of a toxic exposure and provide information of the concentrations of the compound that cause the maximum damage to an organism (Schaefer, 2004). Reproduction tests imply a continuous exposure to a low toxicant dose over a long period of time that may lead to chronic toxicity. Bioaccumulation tests allow the understanding of the pathways and mechanisms trough which a chemical enters the organism and the evaluation of the potential bioaccumulation of the chemical substance (Amorim et al., 2002).. These tests provide important information on the influence of the test chemical on the individual organisms as well as possible ecological impacts on natural populations and communities (Connell, 1999). Therefore, responses from such tests can be extremely useful in environmental protection and management.

Objectives and thesis structure

The main goal of this study was to evaluate the toxicity of different compounds through different levels of effect, such as survival, reproduction and bioaccumulation assays in different test species.

Therefore, the present thesis is organized in two main chapters (papers):

- Chapter I- Ecotoxicology, thesis structure and objectives: describing the objectives and structure of the thesis.
- Chapter II: Review about Personal Care Products (PCP) and their classification.
- Chapter III: "Effect assessment of Triclosan in the terrestrial environment - a soil test battery" (Amorim, M.J.B. , Oliveira, E. and Soares, A.M.V.M. *in prep.*)
- Chapter IV: Review on Polycyclic Aromatic Compounds, its sources, properties and environmental fate.
- Chapter V: "Phenanthrene in the terrestrial environment: reproduction and bioaccumulation assay in *Enchytraeus albidus*" (E. Oliveira, A.S. Teixeira, M.J.B. Amorim, C. Gravato, L. Guilhermino, A.M.V.M. Soares., *in prep.)*.

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CHAPTER II

Review about Personal Care Products (PCP) and their classification

Introduction to Personal Care Products

In recent years, the environmental occurrence of Personal Care Products (PCPs) has been a source of growing concern and their subtle effect on non-target organisms.

Personal care products are defined as chemicals marketed for direct use by the consumer (excluding over-the-counter medication with documented physiologic effects) and having intended end uses primarily on the human body (products not intended for ingestion, with exception of food supplements) (Daughton and Ternes, 1999). In general, these chemicals are designed to alter odour, appearance, touch, or taste, not displaying significant biochemical activity. Most of these chemicals are used as the active ingredients or preservatives in cosmetics, toiletries, or fragrances. They are not used for treatment of disease, but some may be intended to prevent diseases (e.g., sunscreen agents) (Daughton and Ternes, 1999).

Many of these substances are used in very large quantities frequently more than recommended. Personal care products differ from pharmaceuticals in that large amount that can be directly introduced to the environment. For example, these products can be released directly into recreational waters or volatilized into air (e.g. musks). Because of this direct release they can bypass possible degradation in WWTPs (Daughton and Ternes, 1999).

The increasing usage of PCPs and their appearance in different environmental compartments makes them suitable compounds of study. An overview of the different classes of PCPs is also given in this chapter. Triclosan has an antimicrobial mode of action; and was chosen as our chemical compound due to the lack of information about it's toxicity to terrestrial invertebrate organisms and plants.

PCPs and environment

The vast increase in production and usage of PCPs has contributed to their prevalence in surface waters, a problem that has been exacerbated by existing water treatment facilities that are not designed to eliminate effectively these compounds from waste streams (Daughton and Ternes, 1999; Kolpin et al., 2002). Moreover, studies report detectable concentrations of PCPs and their metabolites in surface water, sewage effluent, soils, sediments, groundwater, and drinking water (Daughton and Ternes, 1999).

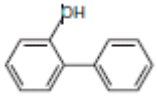
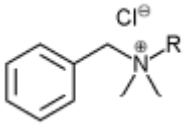
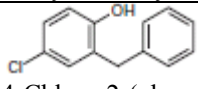
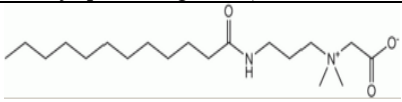
One group of PCP that has received increasing attention is antimicrobials because of their pronounced microbial and algal toxicity and potential for fostering resistance.

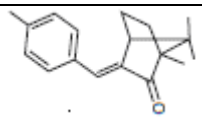
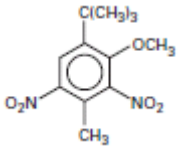
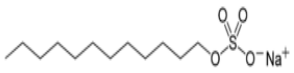
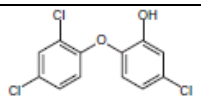
There is a reason to expect that PCPs may have significant impacts on natural biotic communities. For example, widely used antimicrobial agents such as those found in hand soaps and toothpastes are typically designed to kill or to inhibit the growth of a wide range of "undesirable" microbial species. Such broad-spectrum biological activity potentially could cause unintended impacts on sensitive, co-occurring non-target organisms in the residence community. Moreover, individual chemical compounds potentially can interact synergistically or antagonistically with other chemical agents or stressors that may be present in the affected environment. As such, many drugs are taken for very long periods, sometimes a good portion of the user's lifetime (Daughton and Ternes, 1999; Boyd et al., 2004)

PCPs outline

A list of the most commonly identified in environmental samples PCPs is present in table 1, including some information's about: chemical names, structure, and some representative environmental occurrence/effects data. These chemicals, together with their synthetic precursors and transformation products, are continually released into the environment in enormous quantities in enormous quantities as a result of their manufacture, use (via excretion, mainly in urine and feces), and disposal of unused/unwanted drugs and those that have expired, both directly into the domestic sewage system and via burial and landfills.

Table 1 PCPs compounds most commonly identified in environmental samples, their chemical structure, use/origin and trade names.

Compound	**Structure and CAS name**	**CAS RN MW Formula**	**Use/origin**	**Trade names and comments**
Biphenylol	 2-Biphenylol	90-43-7 170.21 $C_{12}H_{10}O$	Antiseptic, fungicide	e.g., Dowicide A
Benzalkonium chloride	 benzyl-dimethyl-tridecyl-azanium chloride	8001-54-5 (mixture) $C_{22}H_{40}ClN$	Antiseptic	Considered one of the safest synthetic biocides known, and has a long history of efficacious use
Chlorophene	 4-Chloro-2-(phenylmethyl)phenol; (*o*-Benzyl-*p*-chlorophenol)	120-32-1 218.68 $C_{13}H_{11}ClO$	Antiseptic	e.g., Santophen 1
Cocamidopropyl betaine	 {[3-(dodecanoylamino)propyl](dimethyl)ammonio}acetate	86438-79-1 $C_{19}H_{38}N_2O_2$	Surfactant, antiseptic	Is a very mild surfactant which does not irritate skin or mucous membranes.

Methylbenzylidene Camphor	 3-(4-Methylbenzyliden) camphor	36861-47-9 254.37 $C_{18}H_{22}O$	Sunscreen agent	e.g., Eusolex 6300
Musk ambrette (a nitro musk)	 2,6-Dinitro-3-methoxy-4-*tert*-butyl toluene	83-66-9 268.27 $C_{12}H_{16}N_2O_5$	Synthetic musk	The nitro musks are being phased out of use in many parts of the world because of toxicity concerns.
Octanol	 1-octanol	111-87-5 130.23 $C_8H_{18}O$	Essential oils	Experimental medical applications utilizing octanol to control certain types of involuntary tremors.
Sodium dodecyl sulphate (SDS)		151-21-3 288.38 $NaC_{12}H_{25}SO_4$	Detergent surfactants	e.g., Sodium lauryl sulphate, sodium salt
Triclosan	 5-Chloro-2-(2,4-dichloro-phenoxy)phenol	3380-34-5 289.54 $C_{12}H_7Cl_3O_2$	Antiseptic	e.g., Irgasan DP 300

Aquatic and terrestrial compartments

The majority of PCPs introduced into the environment is undoubtedly into aquatic systems; the terrestrial environment receives only a secondary input. Although, the primary source for terrestrial environment is probably from disposal of biosolids from WWTPs (Jones-Lepp and Stevens, 2007).

Compounds surviving the various phases of metabolism and other degradative/sequestering actions (i. e., display environmental persistence) can then pose an exposure risk for organisms in the environment. Some degradation products can even be more bioactive than the parent compound (Boyd et al., 2003).

The introduction of PCPs into the environment is partly a function of the quantity of PPCPs manufactured the dosage frequency and amount. The processed liquid effluents from primary and secondary treatments are then discharged to surface waters and the residual solid (sludge) to landfills forms; land disposal, then creates the potential for introduction into groundwater, surface waters or also in drinking waters. Due to their release to the environment they may be present in:

Landfills: PCPs can be introduced to landfills both directly via domestic and industrial routes and indirectly via sewage sludge (Boyd et al., 2003).

Drinking water: PCPs have been identified in domestic drinking water, in (Boyd, et al., 2003).

Sewage treatment plants: They play a crucial role in the separation of PCPs into two exposures pathways associated with the aquatic and the solid phase. Due to the incomplete removal of PCPs from treatment facilities promote an introduction into the environment, According to Boyd (Boyd et al., 2003), triclosan was found in Louisiana sewage treatment plant effluent at concentrations ranging from 10 to 21ng/L.

Many compounds are introduced in the market each year; some of these drugs are from entirely new classes never seen before by the microbiota of a WWTPs. Each of these presents a new challenge to biodegradation In general, most PCPs resist extensive microbial degradation (e.g. mineralization). Although some parent drugs often show poor solubility in water, leading to preferential sorption to suspended particles, they can thereby sorb to colloids and therefore be discharged in aqueous effluent (Daughton and Ternes, 1999).

PCP classes

PCP compounds can be divided in several classes (Daughton and Ternes, 1999), for example:

- Fragances (e.g. nitro and polycyclic musks)
- Antimicrobial compounds (e.g. triclosan)
- UV blockers (e.g. methylbenzylidine camphor)
- Antioxidants and preservatives (e.g. phenols and parabens)
- Insect repellents (e.g. DEET)

Synthetic musk fragances

Two types of synthetic musk fragrances are widely used in Europe and North America: polycyclic and nitro musks (PNMs). They can be found in almost all consumer products (e.g. perfumes, deodorants, cosmetics and soaps) and are released into wastewater after use, so they are present in the environment due to wastewater discharges and land application of biosolids (Herren and Berset, 2000).

Sunscreen agents

Sunscreen agents are increasingly added (in relative amounts of 0.1-10%) to cosmetics and lotions as protection against harmful UV radiation. Though the high hydrophobicity of many of these compounds (log K_{ow}=5-8) indicates the potential for bioaccumulation, relatively little is known about the occurrence and fate of UV filters in the environment. Several of these compounds show estrogenic activity (Sakkas et al., 2003).

Insect repellents

DEET and Bayrepel are the insect repellents mostly used. They have been widely detected in aquatic systems. From limited toxicity data, it can be inferred that DEET is slightly toxic to aquatic invertebrates, fish, and birds (Trenholm et al., 2006).

Preservatives

Parabens are the most common preservatives used in PCPs, pharmaceuticals and food products. Methylparaben and propylparaben are the most widely used and are normally used together due to their synergistic preservative effects. Parabens exhibit estrogenic behaviour (Canosa et al., 2006).

Antimicrobial compounds

Among the antimicrobials, triclosan (TCS) is the compound which has been used for more than 35 years as an antimicrobial and antifungal agent. It is a common constituent of household and personal care products, including soaps, shampoos, deodorants, cosmetics, disinfectants, and detergents (Adolfsson-Erici et al., 2002). Its concentration in personal care products is typically in the range of 0.1-0.3% (w/w) (Sabaliunas et al., 2003). Triclosan has a molecular weight of 289.6, a logarithm of the octanol-water partition coefficient (log K_{ow}) of 4.8 and a pK_a value of 8.14. It is hydrolytically stable, relatively non-volatile with a vapour pressure of $7x10^{-4}$ Pa at 25ºC and is sparingly soluble in water with a solubility of 12mg/L at 20ºC (Reiss et al., 2002).

A vast literature concerned to the TCS effects on the aquatic organisms exist, as described bellow, but a lack of information about TCS effects in terrestrial organism still persist.

TCS has not been reported to be toxic to mammals; it is toxic to aquatic organisms such as *Daphnia magna* with a 48-h median effective concentration (EC_{50}) of 390µg/L and fish (*Pimephales promelas*) with a 96-h median lethal concentration (LC_{50}) of 260µg/L (Orvos

et al., 2002). It's of major concern that TCS is very toxic to some algae species (eg. *Scenedesmus subspicatus*) with a report no-observed-effect concentration (72-h growth NOEC) of 500ng/L (Orvos et al., 2002, Reiss et al., 2002). Moreover, some recent findings indicate that it blocks bacterial lipid biosynthesis by specifically inhibiting the enzyme enoyl-acyl carrier protein reductase (McMurry et al., 1998) led to concerns of the possible development of bacterial resistance to TCS.

The primary rout of entrance of TCS in the environment after its use is through discharge of effluent from WWTPs to surface waters and the disposal of sludge on landfills/farms. Triclosan is present in the raw wastewater of WWTPs and in the effluent due to its incomplete removal in WWTPs, as well as in the sludge generated in WWTPs due to its hydrophobic nature (McAvoy et al., 2002; Reiss, Mackay et al., 2002; Singer et al., 2002; Bester, 2003).

Triclosan in effluents

Triclosan concentrations in effluents of different countries have been reported. For example, in (Agüera et al., 2003) they found TCS concentrations varying from 400ng/L in April and 800ng/L in May to 22100ng/L in June and 19600ng/L in July, during wastewater samplings in Spain during 4 months. This large variation may be related to the difference in input load. On the other hand, the TCS concentrations in the effluents of seven locations in Switzerland WWTPs were found to range from 42ng/L to 213ng/L with an average concentration of 116ng/L.

Large variation in TCS concentration was reported in WWTP effluents from United States and United Kingdom (McAvoy et al., 2002; Reiss et al., 2002; Sabaliunas et al., 2003). The reported TCS concentrations varied from 35ng/L to 2700ng/L with an average of 1180ng/L in USA (n=5) (McAvoy et al, 2002; Halden and Paul, 2005), and from 340ng/L to 1100 ng/L with an average of 753ng/L in U.K. (Sabaliunas et al, 2003). The concentrations of TCS in the final effluents from two WWTPs in USA using activated sludge technology ranged between 240ng/L and 410ng/L, which is much lower than in the effluents from the other two WWTPs using trickling filter technology (ranged from 1600ng/L to 2700ng/L) according to (McAvoy et al, 2002)

Triclosan in biosolis

Sludge samples from 21 plants in Germany were analyzed and are described in (Bester, 2003); the TCS levels ranging between 0.4 mg/kg to 12mg/kg. The primary, secondary and

digested sludge samples from four WWTPs in the USA were analysed in (McAvoy et al., 2002) and TCS concentrations ranged between 0.53mg/kg and 15.6mg/kg and average of 6.97 mg/kg (n=10). They also found that little removal of TCS occurred during anaerobic digestion. Based on systematic data on balances of TCS in a German WWTP (Bester, 2003), about 30% of TCS was adsorbed onto the sludge. This can be explained by its hydrophobic nature resulting in a sorption coefficient (*K*d) of 22,000L/kg in a deactivated and an organic carbon-normalized sorption coefficient (K_{oc}) of 48,000 L/kg (the organic content in sludge was 45%) (Reiss et al., 2002). In Australia, TCS in biosolids ranged from 0.09mg/kg to 16.79mg/kg with an average concentration of 5.58mg/kg on dry weight basis. More than 70% of biosolids are applied on agricultural land, therefore application of biosolids on land could lead to TCS contamination of soil. Those concentrations found for biosolids can worrisome because they are quite high and possibly may affect several terrestrial organisms.

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Chapter III

Effect assessment of Triclosan in the terrestrial environment - a soil test battery

Effect assessment of triclosan in the terrestrial environment - a soil test battery

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Abstract

Triclosan (TCS) is an antimicrobial and preservative agent used as a broad-spectrum bacteriostatic. It can be found in personal-care products (PCPs) such as toothpastes, soaps, cosmetics and even in children's toys. It can be easily spread in the environment by household effluents and consequently in sewage treatment facilities. The main purpose of this study was to assess the effect of TCS in the terrestrial environment. A battery of soil test species, belonging to different trophic levels was used. Standard toxicity tests were performed in the terrestrial organisms *Eisenia andrei*, *Enchytraeus albidus* and *Folsomia candida,* evaluating the survival and reproduction, and in two terrestrial plants *Triticum aestivum* (monocotyledonous) and *Brassica rapa* (dicotyledonous), assessing the emergence and growth. Results showed that *Eisenia andrei* was the most sensitive specie, with species chronic sensitivity decreasing from *E. andrei* > *B. rapa* > *E. albidus* > *F. candida* > *T. aestivum*. The overall results from the selected test battery showed the importance of including different trophic levels due to the different species sensitivities and chemicals mode of action. Moreover, also the use of chronic endpoints is recommended.

Keywords: Personal Care Products (PCPs); plants, earthworms, enchytraeids, collembolans

Introduction

Contamination of the environment occurs by various sources of pollutants and represents a serious problem. For instance, personal care products reach the environment and can be found in significant concentrations in surface and ground waters as well as in soils. Among the personal care products (PCPs) of concern, Triclosan (TCS) has already been the subject of various scientific and regulatory discussions in the recent years the European Community and other countries (Dayan 2007). Triclosan is a broad-spectrum antimicrobial used in a variety of consumer products including toothpaste, shampoos, deodorants, skin lotions and hand soaps as well as in household cleaners and even in textiles (sportswear, bed cloths, shoes, carpets) and children's toys (Singer et al. 2002). It's concentration in these products is usually in the range of 0.1% to 0.3% (Sabaliunas et al. 2003). TCS is washed or rinsed off and may enter the environment via local water waste treatment plants (WWTPs) where typically 90% to 98% is removed as a result of biodegradation and sorption (Orvos et al. 2002; Singer et al. 2002; Kolpin et al. 2002). Nevertheless, U.S. monitoring survey revealed that TCS was commonly detected in surface water at a frequency of 57.6% and at concentrations as high as 2.7μg/L (Kolpin et al. 2002; Reiss et al. 2002). Additional problems may occur due to the formation of the potential biotransformation product Methyl-triclosan (M-TCS), a metabolite of TCS, more lipophilic and environmentally persistent than the parent compound as reported in some studies (Bester 2003; Balmer et al. 2004). Concentrations of M-TCS are generally higher in WWTPs effluent than influent, indicating formation of this transformation product in the treatment process. Despite this, it has been extensively used for human safety in consumer products at low concentrations (not causing carcinogenic or teratogenic effects) (Bhargava and Leonard 1996). Nevertheless, even if non toxic to mammals at the commercial dosages, adverse effects on water organisms (e.g. algae, daphnids) have been reported (Orvos et al. 2002; Wilson et al. 2003; Stephanie L. Fraker 2004), where TCS and it's methylated form also tend to bioaccumulate in organisms such as fish and water plants (Orvos et al. 2002). TCS is also known to adsorb to soil and sediment and it is highly resistant to biodegradation (Ying et al. 2007) but terrestrial toxicity data for TCS is lacking. Therefore, in the present study, the effects of TCS in the terrestrial environment were assessed using a battery of bioassays on non-target soil test species. Standard test species from different trophic levels were selected, which included plants (*Triticum aestivum*, *Brassica rapa*), worms (*Eisenia andrei*, *Enchytraeus albidus*) and collembolans (*Folsomia candida*).

Materials and methods

Test species

Plants

Seeds of two plant test species were used: the monocotyledonous *Triticum aestivum* (local commercial supplier, Aveiro, Portugal) and the dicotyledonous *Brassica rapa* (Carolina Biological Supply Company, US).

Earthworms

Organisms from the test specie *Eisenia andrei* (Oligochaeta) were used. Worms were maintained in culture in laboratory in a moist substrate (mixture of 50% horse manure-50% peat, pH adjusted to 6) at 20±2ºC and a 16:8h light:dark cycle (OECD 1984) and fed once a week with powdered horse manure.

Enchytraeids

The organisms used belong to the test specie *Enchytraeus albidus* (Oligochaeta), Henle 1837. Organisms were cultured in laboratory, kept at 18°C, in a 16:8h light:dark cycle, and fed once a week with finely ground and autoclaved rolled oats (Cimarrom, Portugal).

Collembolans

The standard test specie *Folsomia candida* (Insecta), was used. Organisms were kept in culture in a moist substrate of plaster of Paris and activated charcoal, at 18ºC in the dark and fed weekly on dried baker's yeast (*Saccharomyces cerevisae*).

Test substance

The test substance used, Triclosan (Irgasan, 5-Chloro-2-(2,4-dichlorophenoxy)phenol, $C_{12}H_7Cl_3O_2$, ≥97.0% purity (HPLC)), was purchased from Sigma – Aldrich.

Test soil

All the bioassays were performed with the natural standard soil LUFA 2.2, original from Speyer, Germany (Lokke and Van Gestel 1998). The main characteristics of the test soil are presented in Table 2.

Table 2: Main characteristics of the natural standard soil LUFA 2.2, showing the pH, Organic Matter content (OM), Carbon-Nitrogen ratio (C/N), grain size distribution, Cation Exchange Capacity (CEC) and maximum Water Holding Capacity (WHC).

Soil	*pH ($CaCl_2$)*	*OM (%)*	*C/N*	*Clay (%)*	*Silt (%)*	*Sand (%)*	*CEC (cmol/kg)*	*WHC (%)*
LUFA 2.2	5.8	4.4	14.0	6.0	17.0	77.0	11.2	55.0

Spiking of the soil was done by adding the TCS solved in acetone, as a solution, into the pre-moistened soil batch. Each test concentration was mixed into the whole batch of soil and homogeneously mixed. In addition to the control soil (only water added), a control solvent (acetone) was tested in parallel. Soil samples were allowed to evaporate under the fumehood prior testing. Moisture content was adjusted to 40-60% of the WHC and sub-samples of each batch were introduced into the test replicates. Details on the test setup can be seen in Table 3, including the concentration ranges used in each case.

Table 3: Summary information of the tests performed, showing test species, measured endpoints and concentration ranges used.

Test species	**Endpoints**	**Concentrations (mg/kg)**
Triticum aestivum	Emergence and growth	0-100-180-340-600-1000
Brassica rapa	Emergence and growth	0-32-100-320-640-1000
Eisenia andrei	Survival and Biomass	0-180-360-540-720-1080
	Reproduction	0-10-32-100-320-640
Enchytraeus albidus	Survival and Reproduction	0-3.2-10-32-100-320
Folsomia candida	Survival and Reproduction	0-3.2-10-32-100-320

Test procedures

Plants

The test was performed according to the standard guideline ISO 11269-2 (ISO 1995). The test duration was 14 days after, counted after 50% of seeds emergence in the controls. Four replicates per treatments were used. Each replicate is a plastic pot (100 mm ø, 90 mm height) with 400 ± 50 g of soil (moistened up to 40-60% WHC), having a hole with a fiber glass wick, and 10 seeds were placed at a maximum depth of 1 cm from the soil surface. The pot was then placed on a similar pot, with the two sides open. The two-pot set was placed in a tray with water, and the maintenance of soil moisture was accomplished by

capillary action through the fiberglass wick. Bioassays were carried out at 20 ± 3ºC, with an illumination of 10000 lux, in a 14:10h (light:dark) photoperiod. During the first 7 days, germination time of seeds was reported. At test end, growth and biomass were recorded.

Earthworms

Test procedures followed the standard OECD earthworm acute toxicity test (OECD, 1984). Ten adult worms with well-developed *clitellum*, with 300-600mg, were selected and acclimatized prior to the experiment, being then introduced in a glass vessel, each containing 500g of the test soil, moistened to 40-60% WHC. Water was replenished weekly based on weight loss. Four replicates per treatment were used. The test duration was two weeks. Test runs at 20ºC under a 16:8h photoperiod. At the end of the test, organisms were counted and weighed. The endpoints were survival (monitored at day 7 and 14) and biomass (day 14).

The reproduction test followed the standard OECD guideline 222 (OECD, 2004). The differences from the acute test are such as test duration (56 days) and food supply until the fourth week. After four weeks, the adults were removed, counted and weighted, and the soil with the cocoons is left for an extra 4 weeks. At test end (day 56), the juveniles were counted using a warm water bath (following 40°C to 60°C gradient). After a period of about 20 minutes the juvenile worms appear at the soil surface being easily removed and counted.

Enchytraeids

The Enchytraeids test was performed according to the standard guideline (OECD 220 (2004). Ten adult worms, with eggs in the clitellum, were selected and transferred into each test vessel (glass containers of 250ml with 25g of soil (DW), previously moistened to 40-60% of the WHC, plus food). Containers were covered with parafilm with a few holes for airing. Once a week, the animals were fed with oat flakes and the soil water content was replenished. Four replicates were used per concentration. Adults are removed and counted after 3 weeks. After 6 weeks, the juvenile organisms were immobilisized with alcohol and coloured with Bengal red (1% solution in ethanol) which helps to distinguish the juveniles in the soil. Number of juveniles and adults were recorded.

Collembola

Test procedures were as described in the ISO guideline 11267 for *F. candida* (ISO, 1999). Ten organisms with 10 to 12 days old were used per test container, containing the test soil plus food supply. Four replicates per treatment were used. Vessels were covered with parafilm in which a few holes for aeration were made. Food (2 mg of granulated dry yeast) was added at day 14 and water (based on weight loss) was added weekly. After four weeks, the test ended, and each test vessel was filled with distilled water, gently stirred with a spatula, causing floatation of the organisms. Through digital imaging and using appropriate software (SPSS, 1999), organisms were automatically counted.

Statistical analysis

One way ANOVA and Dunnett's post hoc test (SPSS 1997), was performed to analyse differences between control and treatments. In order to analyse if significant differences occurred between control and control solvent, t-test was performed (SPSS 1997). LC_{50}s were calculated through Probit regression (SPSS 2003). EC_{50}s were determined using the most fitting models (four parameter logistic curve and three parameter sigmoidal curve) (SPSS 1997).

Results

All tests were valid, fulfilling the validity criteria defined in each of the respective guidelines.

Terrestrial plants

For *Brassica rapa,* results from the exposure to triclosan can be observed in figure 1 in terms of effects on length, fresh weight and dry weight.

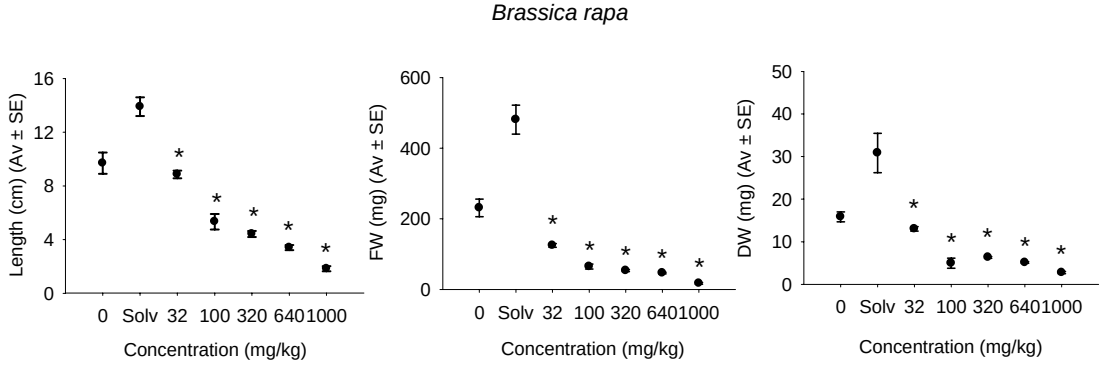

Figure 1: Effect of triclosan in *Brassica rapa* bioassay, showing plant length, fresh weight (FW) and dry weight (DW). Results are expressed as average ± standard error. Asterisks (*) indicate statistically significant differences between control solvent and treatments.

Statistically significant differences occurred between control and control solvent in all measured parameters (T- test, $p<0.05$), therefore, further comparisons were made versus the control solvent. A dose-response effect could be observed within the tested range. Statistically significant differences occurred between control solvent and all the treatments (ANOVA, Dunnett's method; $p<0.05$). EC_{50} and other toxicological values were calculated and are presented in table 4. Seed emergence was checked during the test (Fig. 2).

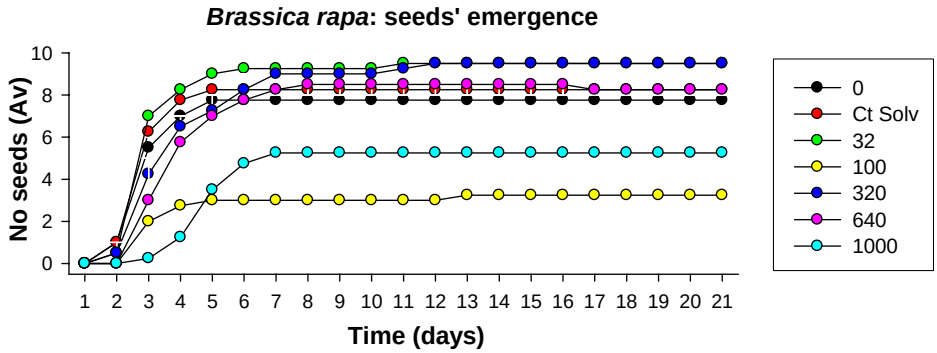

Figure 2 : Effect of triclosan in *Brassica rapa* bioassay, showing emergence of seeds, expressed as average number, from day zero of test setup till test end.
For seed emergence, no significant differences occurred between the control and control solvent at test end. Significant differences occurred only between control and 100mg TCS/kg (soil DW) (ANOVA, Dunnet's; $p<0.05$). In this case only 39% of the seeds emerged. Plants exposed to TCS showed slight signs of clorosis (yellowish colour).

For *Triticum aestivum*, results from the exposure to triclosan can be observed in figure 3 in terms of effects on length, fresh weight and dry weight.

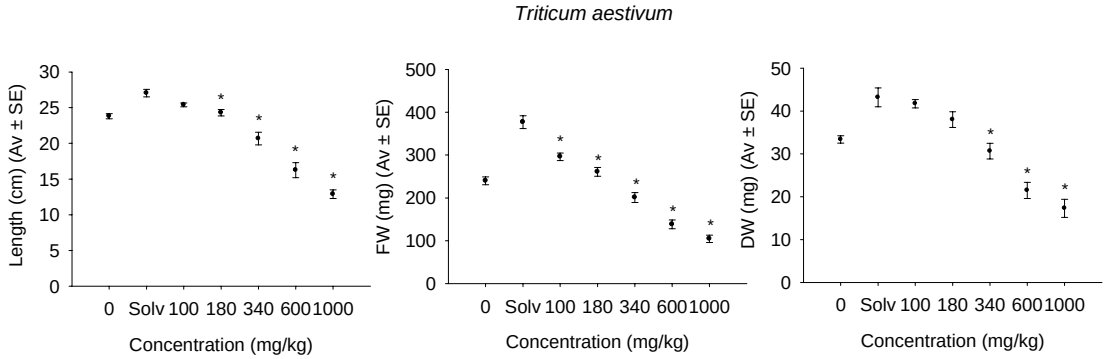

Figure 3 Effect of triclosan in *Triticum aestivum* bioassay, showing plant length, fresh weight (FW) and dry weight (DW). Results are expressed as average ± standard error. Asterisks (*) indicate statistically significant differences between control solvent and treatments.

Statistically significant differences occurred between control and control solvent in all measured parameters (T- test, $p<0.05$), therefore, further comparisons were made versus the control solvent. A dose-response effect could be observed within the tested range. Statistically significant differences occurred between the control solvent and the tested

concentrations: for length with the following concentrations: 180, 340, 600 and 1000mg/kg (ANOVA, Dunnett's Test; $p<0.05$), for the fresh weight with the following concentrations: 100, 180, 340, 600 and 1000mg/kg (ANOVA, Dunnett's Test; $p<0.05$), and for the dry weight with the following: 340, 600 and 1000mg/kg (ANOVA, Dunnett's Test; $p<0.05$). EC_{50} and other toxicological values were calculated and are presented in table 4. Results of seed emergence can be observed in figure 4. Seed emergence was also enhanced by the solvent, but significant differences occurred only for the highest concentration tested (ANOVA, Dunnett's method; $p<0.05$). No morphological changes (e.g. leaf colour or form) were observed.

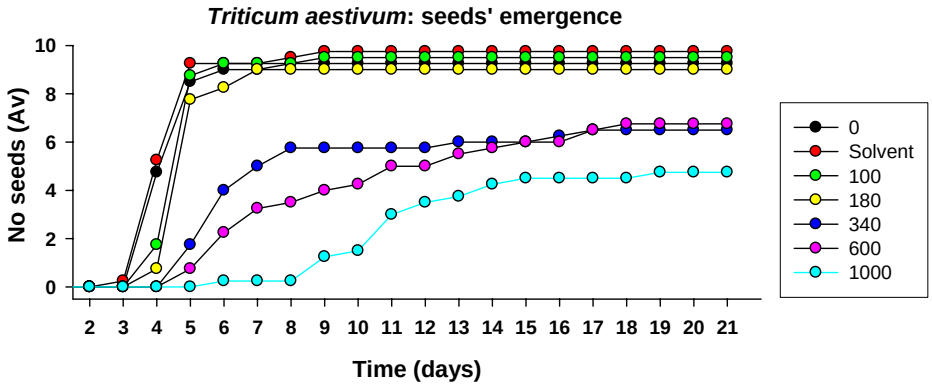

Figure 4 Effect of triclosan in *Triticum aestivum* bioassay, showing emergence of seeds, expressed as average number, from day zero of test setup till test end.

Eisenia andrei

Preliminary tests showed minor effects of the solvent and therefore only this was tested here. Results from the acute exposure of earthworms to triclosan can be observed in figure 5 in terms of effects on biomass.

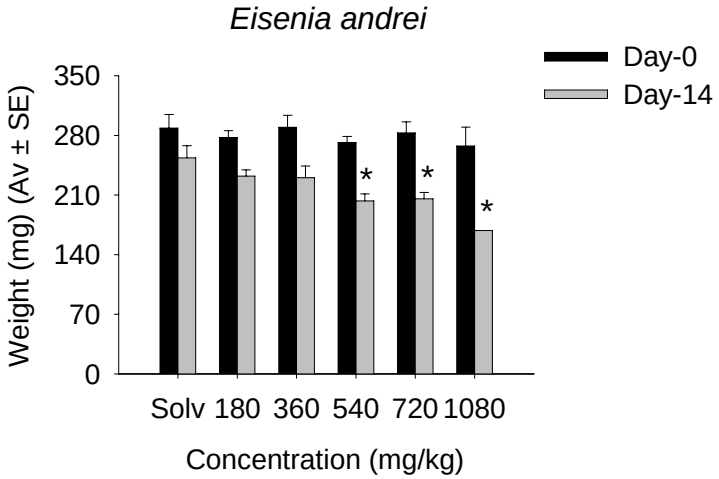

Figure 5 Effect of triclosan in *Eisenia andrei* acute bioassay, showing worms' weight, per number of organisms, at the start and end of the test. Results are presented as average ± standard error. Asterisks (*) indicate statistically significant differences between control and treatments.

Statistically significant differences were observed between the control and the following concentrations: 540, 720 and 1080mg/kg (ANOVA, Dunnett's test, $p<0.05$). Effects on survival were only observed in the higher concentration: 90% mortality at 1080mg/kg, having an LC_{50}=866mg/kg (817<CI<923).

Results from the chronic exposure of earthworms to Triclosan can be observed in figure 6 in terms of effects on biomass, survival and reproduction.

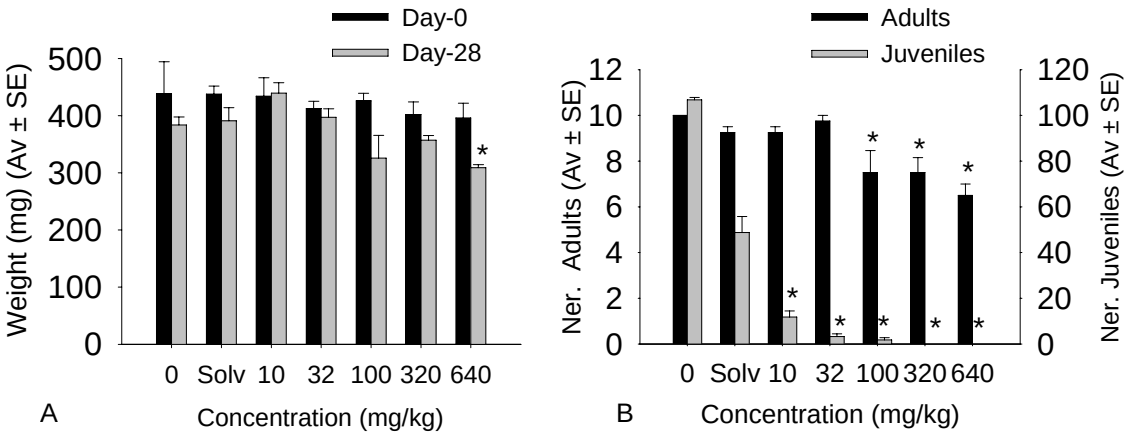

Figure 6 Effect of triclosan in *Eisenia andrei* chronic bioassay, showing **A)** worms' weight, per number of organisms, at the start and day 28 of the test and **B)** number of adults and juveniles at test end. Results are presented as average ± standard error. Asterisks (*) indicate statistically significant differences between control and treatments.

In terms of biomass and number of adults, no differences occurred due to the control solvent (T-test, $p>0.05$), and therefore controls were treated as a pool.

For biomass, only the highest concentrations caused a statistically significant effect (ANOVA, Dunnett's test, $p<0.05$). The number of adults was not very affected either, significant effects were observed for the three highest concentrations (100, 320 and 640mg/kg) (ANOVA, Dunnett's test, $p<0.05$), and LC_{50}=866mg/kg (817<CI<923).

For juveniles, statistically significant differences occurred between control and control solvent (T- test, $p<0.05$), therefore, further comparisons were made versus the control solvent, showing significant differences for all treatments (ANOVA, Dunnett's test, $p<0.05$). A dose-response effect could be observed within the tested range for the number of juveniles showing EC_{50} value of 3.8mg/kg.

Enchytraeus albidus

Results from the chronic exposure of enchytraeids to Triclosan can be observed in figure 7 in terms of effects on survival and reproduction.

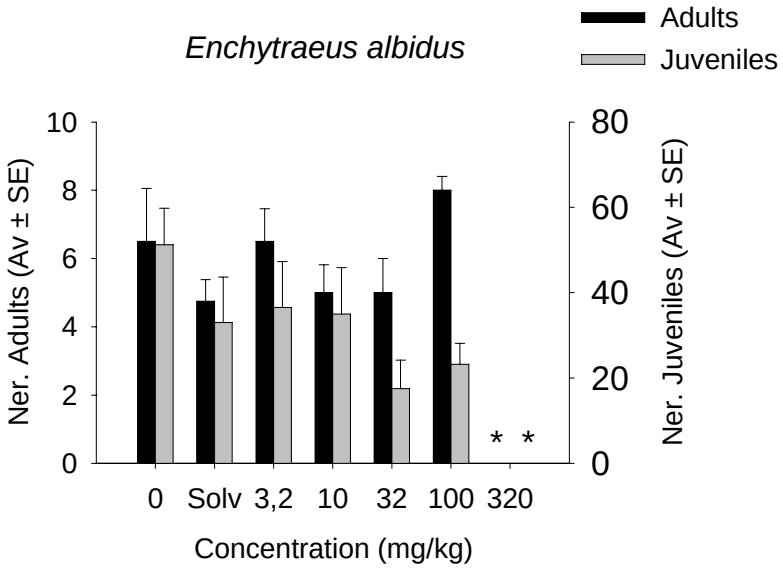

Figure 7 Effects of triclosan in *Enchytraeus albidus* bioassay, showing number of adults and juveniles. Results are presented as average ± standard error. Asterisks (*) indicate statistically significant differences between control and treatments.

For adults and juveniles, no significant differences occurred due to the control solvent (T-test, $p>0.05$), and therefore controls were treated as a pool.

Survival and reproduction were only significantly affected in the highest tested concentration, 320mg/kg (ANOVA, Dunnett's test, $p<0.05$), causing 100% mortality and no reproduction.

Folsomia candida

Results from the chronic exposure of collembolans to triclosan can be observed in figure 8 in terms of effects on survival and reproduction.

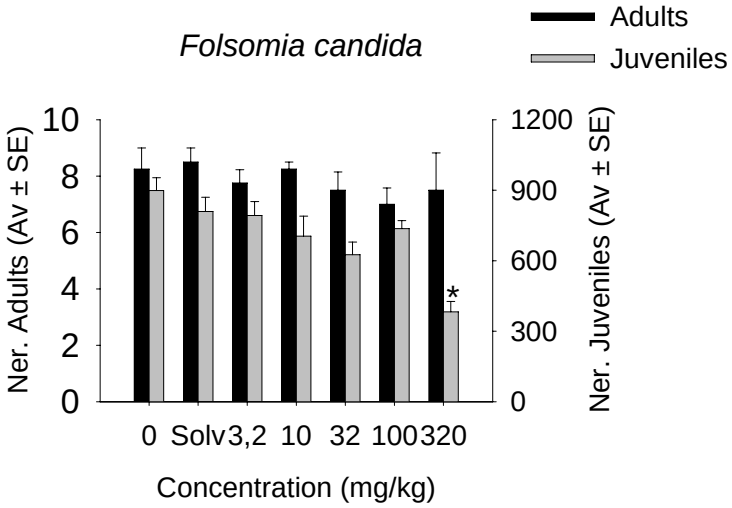

Figure 8 Effects of triclosan on the *Folsomia candida* bioassay, showing number of adults and juveniles at test end. Results are presented as average ± standard error. Asterisks (*) indicate statistically significant differences between control and treatments.

For adults and juveniles, no significant differences occurred due to the control solvent (T-test, $p>0.05$) and controls were treated as a pool. There were no effects on survival within the tested range (ANOVA, $p>0.05$). For juveniles, the effect of triclosan was observed in the highest concentration (ANOVA, $p<0.05$). An EC_{50} value for reproduction of 383.7mg/kg was calculated.

Table 4: Effect concentrations for different test species and different endpoints due to triclosan contamination in soil. The EC_{50}, NOEC and LOEC values are expressed as mg/kg.

Test species	Endpoint	EC_{50} (mg/kg)	CI	NOEC (mg/kg)	LOEC (mg/kg)
Brassica rapa	Length	75.9+	R^2= 0.78; SE=12.2;	<32	32
Brassica rapa	Fresh weight	3.4*	R^2= 0.71; SE=1.3	<32	32
Brassica rapa	Dry weight	25.0*	R^2= 0.82; SE=2.6	<32	32
Triticum aestivum	Length	531.2*	R^2= 0.63; SE=182.9	<100	100
Triticum aestivum	Fresh weight	395.2*	R^2= 0.62; SE=214.4	<100	100
Triticum aestivum	Dry weight	377.9*	R^2= 0.9; SE=32.2	180	340
Acute test					
Eisenia andrei	Biomass loss	-		360	540
Eisenia andrei	No adults	866.0++	817<CI<923	720	1080
Chronic test					
Eisenia andrei	No adults	-		32	100
	No juveniles	3.8*	R^2= 0.76; SE=1.5	<10	10
Enchytraeus albidus	No adults	222,91+	R^2= 0.55;	100	320

			SE=46502120480,3		
Enchytraeus albidus	No juveniles	43,70+	R^2= 0.41; SE=32.3	100	320
Folsomia candida	No adults	>320	-	320	-
Folsomia candida	No juveniles	383.7+	R^2= 0.57; SE=207.0	100	320

* EC_{50} value was calculated using a 4 parameter logistic curve;
+ EC_{50} value was calculated using a 3 parameter sigmoidal curve;
++: EC50 value was calculated using a probit regression;

Discussion

Results of plants revealed different toxicity of TCS for the different test species: *B. rapa* was much more sensitive than *T. aestivum*. For *T. aestivum*, measurements of the different parameters yield similar EC values (app 400mg/kg). On the other hand, ECs for *B. rapa* varied substantially depending on the measured parameter, with plant fresh weight being the most affected parameter. Such differences between the different parameters may be explained due to the fact that this chemical substance induces changes in the osmotic regulation of dicotyledonous. Interestingly it was observed a slight enhancement of emergence caused by the presence of the solvent in both species. This was observed in the case of the control solvent in comparison with the control and also in the lower concentrations tested. The same was observed in a study by Oliveira et al. (in preparation), and other authors, e.g. Bhattacharya and coauthors (1985) showed that acetone caused an enhancement on the root formation level in *Vigna radiate.* Furthermore, it was observed an effect on the time to emerge which is very clear in the highest concentration tested in both plants. In terms of sensitivity, the present results in plants are comparable with other studies, where other test species have been tested with TCS (Samsoe-Petersen et al., 2003), and the most sensitive was a cucumber showing a NOEC for seedling growth similar to *T. aestivum*. Additionally, similarly to the present study, different parameters in one plant have different sensitivity, confirmed in a study also with cucumber (Swchwab and Heim, 1997), where a NOEC>1000 mg/kg was observed for percentage emergence, shoot length, shoot weight and root weight.

Results of earthworms' bioassays showed low effect at the acute level: small decrease in the biomass at the higher concentrations and for survival a NOEC up to 720mg/kg, and an LC50 of 866mg/kg. Results from (Mones and Reiss, 2001), showed that TCS was not acutely toxic for *Eisenia fetida* up to a concentration of 1026mg/kg, a value higher than the one obtained in the present study. On the other hand, there was a high impact at the reproduction level, causing an EC_{50} of app 4mg/kg. This shows that this PCP has high impact at the reproduction or the juveniles. This enhances the importance of including

different effect levels when studying effects of compounds that can be highly underestimated if chronic effects are not taken into account.

Results of enchytraeids' bioassay, showed that the effects at the acute and chronic level were similar, having a 100% mortality and no reproduction at 320mg/kg. It is not so clear if at the concentration of 320mg/kg there are effects at the reproduction level, since the major effect is on the adults, affecting survival primarily and not their ability to reproduce. Nevertheless, effects were more severe at the reproduction (Tab. 3).

Results of collembolans' bioassay showed that at 380mg/kg there was an effect of 50% on reproduction, but no significant effect on the survival within the tested range.

The overall results from the selected test battery of bioassays showed the importance of including different trophic levels, not using assays of a single group of organisms, due to the different species sensitivities and chemicals mode of action.

As could be observed from results in table 4, *Eisenia andrei* was the most sensitive specie, with species chronic sensitivity decreasing in the following order: *E. andrei* > *B. rapa* > *E. albidus* > *F. candida* > *T. aestivum*. Furthermore, results in the terrestrial invertebrates (*E. andrei, E. albidus* and *F. candida*) showed, as expected, that acute parameters are much less sensitive than chronic, with EC50s differing of LC50s in several orders of magnitude.

In comparison to the water contamination and effects in aquatic organisms, these are in general more affected than soil organisms. Among the aquatic organisms (Tab. 4), algae seem to be the most affected group, with an EC_{50} for *Scenedesmus subspicatus* of 1.4µg/L (Reiss et al. 2002). For invertebrate and fish higher values were obtained: e.g. EC_{50} of 343.8µg/L for *Daphnia magna* (Orvos et al. 2002) and NOEC of 200µg/L for *Danio rerio* (Tatarazako et al. 2004). An other study with *Danio rerio* by Oliveira et al. (in preparation) TCS caused significant embryo mortality at 0.5mg/L and based on further results, concentrations higher or equal to 0.3 mg/L seem to pose high risk for the environment.

According to the risk assessment conducted by the Danish EPA (Samsoe-Petersen L, 2003), the PEC values for Triclosan were estimated for activated sludge: PECsoil=0.00004-0.0056mg/kg soil and for "Bio-filter" sludge: PECsoil=0.0005-0.021mg/kg soil. The PNEC value used for the terrestrial risk assessment is PNEC(soil)=0.096µg/kg. The calculation of this PNEC for the soil compartment was based on very few data and was considered preliminary. Considering that TCS may enter the terrestrial environment in concentrations that range from 0.09mg/kg to 16.79mg/kg (Ying and Kookana, 2007, Bester, 2003, McAvoy et al., 2002) and given the results in the present study (specially in *B. rapa* and *E. andrei*, the most affected species), TCS may pose an ecological problem in the environment.

Conclusion

This study represents an important contribution in the assessment of the effect of triclosan in the terrestrial environment, where very limited data existed. Additionally, it shows the importance of using a test battery for effect assessment due to species different sensitivity and that chronic parameters are preferential. Despite the fact that soil organisms are less affected than aquatic, such effects should not be underestimated and may have an impact on the terrestrial ecosystem. Furthermore, the available information on the concentration of TCS may be underestimated at present, given the known continuous entrance in the environment.

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Chapter IV

Polycyclic Aromatic Hydrocarbons, its sources, properties and environmental fate – a review.

Introduction- Polycyclic Aromatic Hydrocarbons

Polycyclic Aromatic Compounds, commonly designated by PAHs, have received much attention since they were found in soils for the first time (Blumer, 1961). Toxicity, environmental persistence and widespread occurrence have made PAHs a pollutant class of global concern (Hafner et al., 2005).

PAHs constitute a variable group of compounds, all being made up of two to six fused aromatic rings in a linear, angular or cluster arrangement and contain by definition only carbon and hydrogen atoms. PAHs are released due to both natural and man-made processes, for example, burning of biomass or fossil fuels, and they are widespread in the environment (Bispo et al., 1999). They are usually generated under inefficient combustion conditions, such as insufficient oxygen (Nam et al., 2003) by primary natural sources which are forest fires and volcanic activity (less important), but most of the PAHs released into the environment arise from anthropogenic sources such as burning of fossil fuels, petroleum refinery, industrial processes, as a constituent of coal tar and motor vehicle exhaust. The lighter PAH (2-3 rings) which are generally not carcinogenic, are mostly found in the gas phase while the heavier ones are mainly associated with airborne particles. Heavier PAH (with more than three rings) are rapidly attached to existing particles, usually soot particles, by adsorption or combustion upon cooling of fuel gas (Kamens et al., 1995). Although these lighter compounds have weaker carcinogenic/mutagenic properties, they are the most abundant in the urban atmosphere and react with other pollutants to form more toxic derivatives (Park et al., 2002). Thus, the implication of human exposure to mixtures of PAHs is of larger relevance than to individual substances.

There are various industrial workplaces for which a significant increase of cancer diseases has been the direct consequence of an unusual high exposure to PAHs. Furthermore, PAH exposure is high in coke plants, coal tar and pitch producing and manufacturing industries, aluminum plants, iron and steel foundries, creosote-rubber-, mineral oil, soot and carbon black producing or manufacturing companies. As highly exposed occupational groups, chimney sweeps, roadmen (pavement-tarring) and roofs (roof-tarring) are also under increased risk (Jacob and Seidel, 2002).

Interestingly, studies of ice cores in Greenland have shown that the atmosphere level of PAHs is now approximately 100 times the level in the period 1500-1799 (Kawamura et al., 1994).

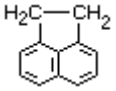
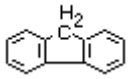
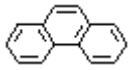
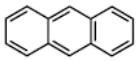
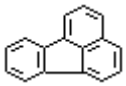
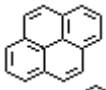
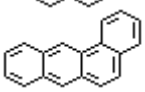
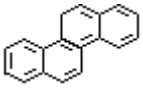
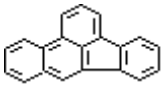
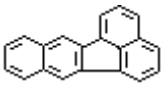
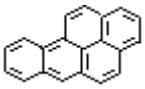
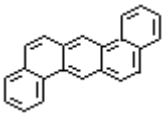
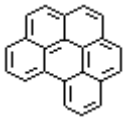
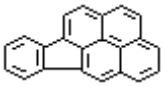
Once these compounds enter in the soil they accumulate in horizons rich in organic matter where they are likely to be retained for many years due to their persistence, high hydrophobicity (Krauss et al., 2000) and low water solubility.

Properties and environmental fate

There are 16 PAHs that are defined as "priority toxic pollutants" by the US Environmental Protection Agency (US-EPA) as shown on Table 5 (ASTDR 2000). Eight of the PAHs that are typically considered as possible carcinogens (CAR-PAHs) are: benzo(a)anthracene, chysene, benzo(b)fluoranthene, benzo(k)fluoranthene, benzo(a)pyrene, dibenzo(a,h)anthracene, indenol(1,2,3-cd)pyrene and benzo(g,h,i)perylene (Menzie et al., 1992). In particular, benzo(a)pyrene has been identified as being highly carcinogenic (Wang et al., 2002). Individual PAHs differ considerably in their physicochemical properties table 5. Low molecular weight (LMW) PAHs are more water soluble and volatile than the high molecular weight (HMW), while the HMW PAHs show higher hydrophobicity than the LMW compounds. The difference in hydrophobicity is also observed by the octanol-water partitioning coefficient (KOW) shown in Table 5. These physico-chemical properties largely determine the environmental behaviour of PAHs, and indicate that transfer and turnover will be more rapid for LMW PAHs than for the heavier PAHs.

The semi-volatile nature of the LMW PAHs means that they exist in the atmosphere partly as vapours and are therefore highly susceptible to atmospheric degradation processes. The HMW PAHs, on the other hand, are primarily associated with particles in the atmosphere and water, and are therefore less available for degradation (ASTDR 2000).

Table 5 Main physical-chemical characteristics of 16 Polycyclic Aromatic Hidrocarbons (PAH) defined as "priority toxic pollutants" by the US Environmental Protection Agency (US-EPA).

PAHs	Molecular formula	No. rings	Log Kow[a]	Relative molecular mass[a]	Geno toxicity[a]	Carcino genicity[a]	Aquous solubility (mg/L)
Naphthalene		2	3,4	128,2	-	(?)	31
Acenaphthylene		3	4,1	152,2	(?)	No studies	16
Acenaphtene		3	3,9	154,2	(?)	(?)	3,8
Fluorene		3	4,2	166,2	-	-	1,9
Phenanthrene		3	4,6	178,2	(?)	(?)	1,1
Anthracene		3	4,5	178,2	-	-	0,045
Fluoranthene		4	5,2	202,3	+	(+)	0,26
Pyrene		4	5,2	202,3	(?)	(?)	0,13
Benzo(a)anthracene		4	5,6	228,3	+	+	0.011
Chrysene		4	5,9	228,3	+	+	0.006
Benzo(b)fluoranthene		5	6,1	252,3	+	+	0.0015
Benzo(k)fluoranthene		5	6,8	252,3	+	+	0.0008
Benzo(a)pyrene		5	6,5	252,3	+	+	0.0038
Dibenzo(ah)anthracene		5	6,5	278,4	+	+	0.0006
Benzo(ghi)perylene		6	7,1	276,3	+	-	0.0003
Indeno(1,2,3-cd)pyrene		6	6,6	276,3	+	+	0.0002

[a] Octanol-water partition coefficients, relative molecular mass, genotoxicity, carcinogenicity: data from Environmental Health Criteria 202 (IPCS, 1998).
[b] (Sverdrup et al., 2003)
(+) positive; -, negative; ?, questionable; parentheses, result from small database.

PAHs can also be found in water: Benzo(a)pyrene (BaP), Phenanthrene (PHE), Chysene, Benzo(b)fluoranthene and Benzo(k)flouranthene appear in high concentration in water, as reported by (Anyakora et al., 2005)

PAHs in soil

In soil most PAHs are strongly sorbed to the organic matter, making them relatively unavailable for degradation processes (Wilcke, 2000). PAHs can therefore remain in the soil for many centuries, posing a long-term threat to the environment, although LMW PAHs are partly lost through degradation processes, volatilization and leaching (Johnson et al., 2002). The effect of sorption generally increases as the number of benzene rings in the PAH molecule increases, since this implies higher lipophilicity. Furthermore, it has been shown that the degradability and extractability of organic compounds in soil decreases with the time they have been in contact with the soil: a phenomenon referred to as ‘aging’ or ‘weathering’ (Johnson et al., 2002).

Toxicity of PAHs

A wide range of PAHs has been reported to induce ecotoxicological effects in a diverse group of biota, such as microorganisms, terrestrial plants, aquatic and soil invertebrates. In order to define a priority study list among the different existing PAHs, a literature research was conducted, taking into account the PAH studied, the organisms, the endpoints evaluated and the effect concentrations on organisms (table 6).

Table 6 **Data on toxicity of polycyclic aromatic hydrocarbons resulting from literature research.** The diminutives in the toxic column corresponds to the following: Ant- anthracene; B(a)A-Benzo(a)Anthracene; B(a)P- Benzo-(a)-pyrene; Fluo-Fluoranthene; Naph-Naphthalene; Phe-Phenanthrene and Pyr-Pyrene.

Toxic	Test organisms[a]	Tested concentrations (mg/kg (DW))	Estimated values	Results	References
Ant	*Eisenia fetida*	200, 500, 1000	Survival (%)	84, 87, 86%	(Contreras-Ramos et al., 2006)
B(a)A	*Aporrectodea longa*	3.6 mg/kg	extractability/availability	Both reduced	(Johnson et al., 2002)
B(a)P	*Trifolium pretense*	1, 10, 100, 500	NOEC	>470mg/kg (dw)	(Sverdrup et al., 2007)
B(a)P	*Lolium perenne*	1, 10, 100, 500	NOEC	>470mg/kg (dw)	(Sverdrup et al., 2007)
B(a)P	*Brassica alba*	1, 10, 100, 500	NOEC and LOEC	86 and 470mg/kg (dw)	(Sverdrup et al., 2007)
B(a)P	*Enchytraeus crypticus*	1, 3, 10, 30, 100, 300, 1000	NOEC	>947 mg/kg (dw)	(Sverdrup et al., 2007)
B(a)P	*Hypoaspis aculeifer*	1, 3, 10, 30, 100, 300, 1000	NOEC	>947 mg/kg (dw)	(Sverdrup et al., 2007)
B(a)P	Soil nitrification	1, 3, 10, 30, 100, 300, 1000, 3000	NOEC and LOEC	293, 977mg/kg (dw)	(Sverdrup et al., 2007)
B(a)P	*Eisenia fetida*	50ug, 1mg, 100mg, 1000	LOEC	50ug/kg	(Saint-Denis et al., 2000)
B(a)P	*Eisenia fetida*	50, 100, 150	Survival (%)	86, 79, 84%	(Contreras-Ramos et al., 2006)
B(a)P	Micro-organisms	50, 100, 150	Toxic removal %	13%	(Contreras-Ramos et al., 2006)
B(a)P	*Eisenia fetida*	50ug, 1mg, 100mg, 1000	LOEC	50µg/kg soil	(Saint-Denis et al., 1999)
Fluo	*Daphnia magna*	0.040-1.2 µg/L	EC_{05}	39 ug/L	(Olmstead and LeBlanc, 2005)
Fluo	*Daphnia magna*	1, 10, 100µg/L	EC_{50}	194 +/- 11 ug/L	(Olmstead and LeBlanc, 2005)
Fluo	*Sinapsis alba*	10, 100, 1000	EC_{20} seedling growth	650mg/kg (dw)	(Sverdrup et al., 2003)
Fluo	*Trifolium pretense*	10, 100, 1000	EC_{20} seedling growth	140mg/kg (dw)	(Sverdrup et al., 2003)
Fluo	*Lolium perenne*	10, 100, 1000	EC_{20} seedling growth	490mg/kg (dw)	(Sverdrup et al., 2003)
Fluo	*Folsomia fimetaria*	0, 15, 30, 60, 120, 240	LC_{50}	81mg/kg (dw)	(Sjursen et al., 2001)
Fluo	*Eisenia veneta*	0, 10, 30, 100, 300, 1000	LC_{50}	416mg/kg (dw)	(Sverdrup et al., 2002a)
Fluo	*Enchytraeus crypticus*	0, 10, 20, 40, 80, 160, 320, 640, 1280, 2560	EC_{10}	15mg/kg (dw)	(Sverdrup et al., 2002b)
Fluorene	*Sinapsis alba*	10, 100, 1000	EC_{20} seedling growth	120mg/kg (dw)	(Sverdrup et al., 2003)
Fluorene	*Trifolium pratense*	10, 100, 1000	EC_{20} seedling growth	55mg/kg (dw)	(Sverdrup et al., 2003)
Fluorene	*Lolium perenne*	10, 100, 1000	EC_{20} seedling growth	380mg/kg (dw)	(Sverdrup et al., 2003)
Fluorene	*Folsomia fimetaria*	0, 10, 20, 40, 80, 160	LC_{50}	39mg/kg (dw)	(Sjursen et al., 2001)
Fluorene	*Eisenia veneta*	0, 10, 30, 100, 300	LC_{50}	69mg/kg (dw)	(Sverdrup et al., 2002a)

Table 6 (continued)

Toxic	Test organisms[a]	Tested concentrations (mg/kg (DW))	Estimated values	Results	References
Fluorene	*E. crypticus*	0, 10, 20, 40, 80, 160, 320, 640, 1280, 2560	EC_{10}	25mg/kg (dw)	(Sverdrup et al., 2002b)
Naph	*Daphnia magna*	0.020-0.080 µg/L	EC_{05}	690 ug/L	(Olmstead and LeBlanc, 2005)
Naph	*Daphnia magna*	10, 100, 1000µg/L	EC_{50}	4610 +/- 820 ug/L	(Olmstead and LeBlanc, 2005)
Phe	Micro-organisms	200, 500, 1000	Toxic removal %	77%	(Contreras-Ramos et al., 2006)
Phe	*Daphnia magna*	0.040-0.53 µg/L	EC_{05}	46 ug/L	(Olmstead and LeBlanc, 2005)
Phe	*Daphnia magna*	10, 100, 1000µg/L	EC_{50}	349 +/- 19 ug/L	(Olmstead and LeBlanc, 2005)
Phe	*Sinapsis alba*	1, 10, 100, 1000	EC_{20} seedling growth	77mg/kg (dw)	(Sverdrup et al., 2003)
Phe	*Trifolium pratense*	1, 10, 100, 1000)	EC_{20} seedling growth	37mg/kg (dw)	(Sverdrup et al., 2003)
Phe	*Lolium perenne*	1, 10, 100, 1000	EC_{20} seedling growth	300mg/kg (dw)	(Sverdrup et al., 2003)
Phe	*Eisenia veneta*	0, 10, 30, 100, 300	LC_{50}	134mg/kg (dw)	(Sverdrup et al., 2002a)
Phe	*E. crypticus*	0, 10, 20, 40, 80, 160, 320, 640, 1280, 2560	EC_{10}	40mg/kg (dw)	(Sverdrup et al., 2002b)
Pyr	*Daphnia magna*	0.050-0.84 µg/L	EC_{05}	22 ug/L	(Olmstead and LeBlanc, 2005)
Pyr	*Daphnia magna*	10, 1000µg/L	EC_{50}	72,7 +/- 7,8 ug/L	(Olmstead and LeBlanc, 2005)
Pyr	*Aporrectodea longa*	11mg/kg (dw)	extractability/availability	Both reduced	(Johnson et al., 2002)
Pyr	*Sinapsis alba*	10, 100, 1000	EC_{20} seedling growth	120mg/kg (dw)	(Sverdrup et al., 2003)
Pyr	*Trifolium pratense*	10, 100, 1000	EC_{20} seedling growth	49mg/kg (dw)	(Sverdrup et al., 2003)
Pyr	*Lolium perenne*	10, 100, 1000	EC_{20} seedling growth	>1000mg/kg (dw)	(Sverdrup et al., 2003)
Pyr	*Folsomia fimetaria*	0, 15, 30, 60,120, 240	LC_{50}	53mg/kg (dw)	(Sjursen et al., 2001)
Pyr	*Eisenia veneta*	0, 10, 30, 100, 300	LC_{50}	155mg/kg (dw)	(Sverdrup et al., 2002a)
Pyr	*E. crypticus*	0, 10, 20, 40, 80, 160, 320, 640, 1280, 2560	EC_{10}	11mg/kg (dw)	(Sverdrup et al., 2002b)

An overview of studies with different PAHs is given in Table 6, however is important to take into account the relevance of some particular studies from the table above using terrestrial or aquatic organisms to assess PAHs toxicity, due to their toxicity, persistence in the environment and low solubility in water. The effect concentrations were based on nominal values given in the references.

Contreras-Ramos and co-workers (2006) studied the uptake by *Eisenia fetida* of three PAHs (Phenantrene (PHE), Anthracene (Anthra) and B(a)P) using different concentrations, and have measured the PAHs concentration in tissue and soil exposed during a period of 11 weeks. Anthracene showed no toxicity up to 1000mg/kg and BaP caused no effect on the earthworms' survival up to 150mg/kg. Phenanthrene was the one that affected the animals most: no worms survived at 150mg/kg concentration. It's possible to observe that at the end of 11 weeks there was an uptake of PHE by *Eisenia fetida* for <100mg/kg concentrations. The results were fairly different with the other PAHs. In another study (Olmstead and LeBlanc., 2005), the effect of four PAHs was studied: Pyrene, Naphthalene, Phenanthrene and Fluoranthene, both individually and in mixtures. Tests were performed with *Daphnia magna* using growth rate as parameter. It was noticed that Pyrene and Phenanthrene decrease molt frequency of the daphnids whereas Naphthalene and Fluoranthene did not cause effect. Therefore Pyrene and Phenanthrene may have caused effects on growth rates through a molting dependent pathway that was distinct from the mode of action of naphthalene and fluoranthene. According to single PAH exposures, Pyrene and Fluoranthene reduced growth rate at similar concentrations ranges ≈10-100µg/L. Phenanthrene reduced growth rates at ≈40-400µg/L. Naphthalene was less effective at retarding daphnid growth rates; having effects at concentrations above >1000 µg/L. The effects of BaP were also studied in different organisms (Sverdrup et al., 2007): three species of terrestrial plants (*Trifolium pretense*, *Lolium perene*, and *Brassica alba*), two soil invertebrates (*Enchytraeus crypticu*s and *Hypoaspis aculeifer*.), and the nitrifying ability of soil bacteria. BaP showed lower toxicity to many different soil organisms because of the low water solubility of this substance. LOEC values for soil bacteria and *Brassica alba* were 977 and 470mg/kg, respectively. Several studies report the effects of PAHs (Naph, Anthra, Phe, Pyr, B(a)A and B(a)P) and PACs (Polyclic aromatic compounds) to the springtail *Folsomia fimetaria* (Sjursen et al., 2001; Sverdrup et al., 2001), the Oligochaeta *Enchytraeus crypticus* (Sverdrup et al., 2002b), the earthworm *Eisenia veneta* (Sverdrup et al., 2002a). Tests performed with *Folsomia fimetaria* (Sjursen et al., 2001) , showed

that Fluorene, fluoranthene and pyrene among others PAC, caused a dose-related decrease in drought tolerance in exposed adults and some of the tested substances significantly reduced the drought tolerance of *Folsomia* fimetaria at concentrations that had little effect on survival. The EC10 values for *Folsomia* fimetaria were also estimated in (Sverdrup et al., 2001) for reproductive output, and were for fluoranthene 37mg/kg, fluorine 7.7mg/kg, phenanthrene 23mg/kg and pyrene 10mg/kg.

On the other hand, according to (Sverdrup et al., 2002b), the effects of eight (PACs) on the survival and reproduction of *Enchytraeus crypticus* were investigated. The EC10 values were estimated and were for fluoranthene, 15mg/kg; for fluorine, 25mg/kg; for phenanthrene, 40mg/kg; and for pyrene, 11mg/kg. Enchytraeids generally seem less sensitive than collembolans

The effects of PACs on the survival and growth of the earthworm *Eisenia veneta* are reported in Sverdrup (Sverdrup et al., 2002a). In general, earthworm growth was reduced at PAC concentrations above 25mg/kg soil dry weight.

In a study with *Folsomia candida* (Sjursen et al., 2001), Fluorene was the PAH that shows more toxicity with a LC_{50} values 39mg/kg dry soil.

The effects on the survival and reproduction were assessed in (Sverdrup et al., 2002b) for the oligochaeta *Enchytraeus crypticus*. Lethality was determined by the LC_{50} for different compounds: Pyrene, Fluoranthrene, Phenantherene, Fluorene; the LC_{50} were, >2.300, >2.500,>2.000 and 1.600 (mg/kg) respectively. The NOEC values were also determined 18, 38, 34, and 27 (mg/kg) respectively. The data show that *E. crypticus* are less sensitive to those PAHs than *F. candida*.

Latter in (Sverdrup et al., 2002a) a survival and reproduction test was performed with *Eisenia veneta*. The substances tested were Pyrene, Fluoranthrene, Phenantherene, Fluorene. A summary of the toxicity test results on the earthworm are reported on Table 6. The author concludes that the range in sensitivity for the 3 invertebrates that generally *E. veneta* was slightly less sensitive than *F. fimetaria*, whereas *E. crypticus* was rather insensitive to the substances tested.

This PAHs literature research on its effects on different standard test species was very important for our chemical selection for the sutudy presented in the next chapter. Phenanthrene was selected as our test substance due to the fact that is one of the PAHs that showed more toxicity from the overall studies and it was shown to bioaccumulate in organisms (Hofman et al., 2008). It is among the more soluble PAHs, such as naphthalene, acenaphtylene, or phenanthrene with 2-3 rings, having been found at the

highest concentrations in water extracts. These affected significantly the survival or reproduction of organisms, while PAH congeners of higher lipophilicity did not.

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Chapter V

Phenanthrene in the terrestrial environment: reproduction and bioaccumulation assays in Enchytraeus albidus

Phenanthrene in the terrestrial environment: reproduction and bioaccumulation assay in *Enchytraeus albidus*

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Abstract

Enchytraeids are important members of the soil fauna living in the true soil layer instead of the humus like most earthworms, resulting in a different interaction with chemicals in soil.The main goal of this study was to determine the toxicity of PHE in *Enchytraeus albidus* at different effect levels: survival, reproduction and bioaccumulation, following the standard guidelines. Results show that PHE reduced significantly the reproduction of *E. albidus* within the tested range (EC_{50}=36,8mg/kg). The toxicokinetic behaviour of PHE in *E. albidus* kept in soil was studied. To determine the assimilation and elimination kinetics, the organisms were exposed to contaminated soil for 14 days, followed by a depuration period of 14 days. Equilibrium was achieved within 12 days. Bioaccumulation factors (BAFs) based on the PHE concentrations in soil was calculated and a BAF of 14.3 was obtained, and the uptake and elimination rates were 4.379 g soil/g worm day-1 and 0.305 d^{-1} respectively. *E. albidus* showed to be sensitive to PHE at different toxicity effect levels and study of different endpoints proved to be more reliable.

Keywords: Polycyclic aromatic hydrocarbons, toxicity, Enchytraeid

Introduction

Soils are increasingly becoming sinks of a wide range of hazardous pollutants generated by human activities. Among these are the polycyclic aromatic hydrocarbons (PAHs) such as naphththalene, phenanthrene or benzo[a]pyrene, aromatic compounds coming from coke production, petroleum refining and other high-temperature industrial process (Bispo et al., 1999). Once entered in the soil compartment they tend to accumulate in horizons rich in organic matter where they are likely to be retained for many years due to their persistence and hydrophobicity (Krauss et al., 2000). The lipophilic nature, relatively low mobility and high resistance to degradation of PAHs can result in the bioaccumulation of these chemicals by soil biota (Tarradellas et al., 1982).

Enchytraeids (Oligochaeta) were selected as test organisms due to their important ecological role in the soil compartment (Didden, 1993). *Enchytraeus albidus* (Enchytraeidae) is a standard test species with standardized guideline to assess effects at survival and reproduction level (OECD 220 (2004) and the bioaccumulation potential of chemicals Draft guideline: Bioaccumulation: soil test using terrestrial Oligochaetes). They have been found to contribute significantly to soil respiration, to constitute the second highest biomass in many soils, and to affect cycling of nutrients and community metabolism considerably (Römbke, 1992; Didden, 1993). These worms can accumulate and be affected by a variety of organic and inorganic compounds (Amorim et al., 2002). In addition, this group of worms is an important component in terrestrial food webs. A wide variety of organisms, including: birds, mammals, reptiles, amphibians, fish, insects, nematodes and centipes prey upon enchytraeids. Taking this into consideration, the analysis of the bioaccumulation potential is important for the assessment of exposure (internal concentrations) and food chain transfer within the risk assessment of chemicals (Bruns et al., 2001).

The aim of this study was to assess the toxicity and bioaccumulation of PHE in *E. albidus.* Phenanthrene (three ring PAH) was selected as a model compound for studying bioaccumulation since (i) it is found in high concentrations in PAH contaminated environmental samples (Eom et al., 2007), (ii) many PAHs containing a phenanthrene moiety are carcinogenic (Bezalel et al., 1996); (iii) its hydrophobicity (log k_{ow}=4.6) and persistency in the environment makes it a suitable test substance for bioaccumulation

studies; (iv) it was show to be bioaccumulated (Hofman et al., 2008) and (v) it is soluble in organic solvents, not posing some of the practical problems of other PAHs, (vi) there is a lack in information about PHE effects in soil and its effects in soil organisms.

Material and Methods

Test chemical

Phenanthrene (>98% chemical purity, Sigma Aldrich), a three aromatic rings benzene, was used. PHE has a low vapour pressure, low solubily in water (1.29mg/L), high Log *Kow* (4.46).

Test soil

Experiments were performed with the natural standard soil LUFA 2.2, original from Speyer, Germany (Lokke and Van Gestel, 1998). The main characteristics of the test soil are presented in Table 7.

Table 7 Main characteristics of the natural standard soil LUFA 2.2, showing the pH, Organic Matter content (OM), Carbon-Nitrogen ratio (C/N), grain size distribution, Cation Exchange Capacity (CEC) and maximum Water Holding Capacity (WHC).

Soil	*pH* ($CaCl_2$)	*OM (%)*	*C/N*	*Clay (%)*	*Silt (%)*	*Sand (%)*	*CEC (cmol/kg)*	*WHC (%)*
LUFA 2.2	5.8	4.4	14.0	6.0	17.0	77.0	11.2	55.0

The contamination of the test soil with Phenanthrene was done by homogeneously mixing a solvent (acetone) solution of the chemical into the pre-moistened soil batch. Soil samples were allowed to evaporate the solvent under the fumehood prior testing. Moisture content was adjusted to 40-60% of the WHC and sub-samples of each batch were introduced into the test vessels In addition to the contaminated test soil a control solvent (acetone) was tested in parallel.

Test specie

The organisms used belong to the test species *Enchytraeus albidus* (Oligochaeta), Henle 1837. Organisms were cultured in laboratory, kept at 18°C, in a 16:8h light:dark cycle, and fed once a week with finely ground and autoclaved rolled oats (Cimarrom, Portugal).

Experimental procedures

Reproduction assay

The *Enchytraeids* test was performed according to the standard guideline (OECD 220 (2004). The concentrations tested were: 0, 5, 10, 20, 40, 80 and 160mg/kg. A control with the solvent (acetone) was added. Ten adult worms, with eggs in the clitellum, were selected and transferred into each test vessel (glass containers of 250ml with 25g of soil (DW), previously moistened to 40-60% of the WHC, plus food). Containers were covered with parafilm with a few holes for airing. Once a week, the animals were fed with oat flakes and the soil water content was replenished. Four replicates were used per concentration. Adults are removed and counted after 3 weeks. After 6 weeks, the juvenile organisms were immobilisized with alcohol and coloured with Bengal red (1% solution in ethanol) for counting procedures. Number of juveniles and adults was recorded.

Bioaccumulation test

The bioaccumulation test was performed according to the draft guideline "Bioaccumulation guideline: Soil test using terrestrial Oligochaetes". The experiment includes 14d for the uptake phase plus 14d for the elimination phase. At test start, animals with similar size and with a well developed clitelum were selected and introduced in test vessels with soil spiked with 8mg PHE/kg soil (DW). Food was added and replenished once a week, as well as the soil water content. After 14 days of exposure, animals were transferred into clean soil for a similar period for the elimination phase to take place. Samplings were performed at days 1, 2, 4, 7, 9, 11, and 14 for the uptake phase and at 6h, 1, 2, 4, 7, 10, and 14 days during the elimination phase. Seven replicates were used. At each sampling time, organisms were picked and washed in deionised water, gently dried on filter paper, weighted and frozen with liquid nitrogen. Soil was also sampled and stored in the freezer for further analysis.
Control soil was spiked with acetone, which was left to evaporate under a fume hood.

Phenantrene analysis

Soil: Extraction method

Phenantrene was extracted from soil, through an adaptation of the method described by Song (Song et al., 1995). A sample of 0.5g of soil was weighted into a 15ml tube and 10ml acetone was added, vortexed during 1min and sonicated for 20min. The PHE extracted with acetone was separated from the soil by centrifugation at 4000x g for 15min; the supernatant was sampled into a new tube. The same procedure was repeated five times adding new acetone volume to the soil previously extracted, in order to guarantee a total extraction.

Soil: Gas chromatography-mass spectrometer (GC-MS) analysis

The extracted material was quantified in a gas chromatography-mass spectrometer (Varian, Palo Alto, CA), GC model 4000, mass selective detector model 4000 and a 30 m x 0.25 mm DB5 – 5% phenyl methyl siloxane capillary column. The injector and transfer line temperature were set at 250 ºC, and the temperature program was: 1 min at 50 ºC, then increase to 320 ºC at 50 ºC min^{-1}, maintaining isotherm for 5 min. A 0.1 mL aliquot was injected. The injection was carried out in split mode with an initial split ratio of 30. Then, it was changed to 0, at 0.001 s, to concentrate the desorbed sample. Finally it was set to 80, after 5 min, for fiber cleaning purpose. The carrier gas was Helium N60 (Praxair), at a flow rate of 1 mL min^{-1}. The ion trap detector was set to electron impact (EI) mode, at 70 eV, on full scan mode, with an acquisition range (m/z) from 50 to 205 and an acquisition frequency of 2.38s^{-1}.

The mass selective detector was operated in the full scan mode to obtain respective mass spectrum data for identification of hydrocarbon components. Fluorene was used as internal standard at a concentration of 180 µg L^{-1}. PAH identification was achieved by comparing their linear retention index (LRI) and mass spectra with those of pure standards.

Organisms: Fixed Fluorescence analysis

The Fixed Wavelength Fluorescence method (FF) was used. Fluorescence analyses were performed using a JASCO FP-6200 Spectrofluorometer, coupled to a software package operated by Windows 3.1. A standard curve was drawn for PHE with methanol

(50%) and was used to calculate the concentration in each organism. Briefly, each sample with a group of organisms was digested in a phosphate buffer (pH 7.2) and then diluted in 50% methanol. The solution was screened for Phenanthrene-type metabolites at an excitation wavelength of 256nm and an emission wavelength of 380nm, which is optimal for 3-ring PAH compounds/metabolites. For quality assurance, blanks were run for calibration of samples. The results presented are related to worms' weight.

Data analysis

Reproduction assay

In order to analyse if significant differences occurred between control and control solvent, t-test was performed (SPSS, 1997). One way ANOVA and Dunnett's post hoc test (SPSS, 1997), was performed to analyse differences between control and treatments. EC_{50}, EC_{20} and EC_{10} were calculated through a Logit regression and LC_{50}, value was calculated through a Weibull regression (ToxRat, 2003).

Bioaccumulation assay

The decrease constant of chemical concentration over time during the uptake phase, was calculated assuming a first-order kinetic model (model 1), according to Widianarko and Van Straalen (1996):

$$C(t) = C_0 e^{-k0t} \qquad (1)$$

Where C(t)= concentration in soil (mg/kg), C_0=initial external concentration (mg/kg); and K_0= rate of constant for decrease of the chemical in the medium (day^{-1}).

For the uptake and elimination in the worms the following kinetic models were used: for $t \leq t_c$:

$$Q(t) = \frac{a}{k - k_0}(e^{-k0t} - e^{-ktc}) \qquad (2)$$

and fot $t > t_c$:

$$Q(t) = \frac{a}{k - k_0}(e^{-k0tc} - e^{-ktc})e^{-k(t-tc)}) \qquad (3)$$

where *Q(t)* is the concentration in the organism at time *t*, (ng/mg worm), a is the assimilation rate (ng day^{-1}), *k* is the elimination constant (day^{-1}), k_0 is the soil decay

constant (day^{-1}), t is time (days), t_c is time at which animals were transferred to clean soil (days).

The bioaccumulation factor (BAF) was determined as follows (Belfroid et al.,1993):

$$BAF = \frac{a}{k} \qquad (4)$$

where a is the assimilation constant and k is the elimination rate.

Results

Reproduction assay

Results from the chronic exposure of Enchytraeids to Phenanthrene can be observed in figure 9 in terms of effects on survival and reproduction.

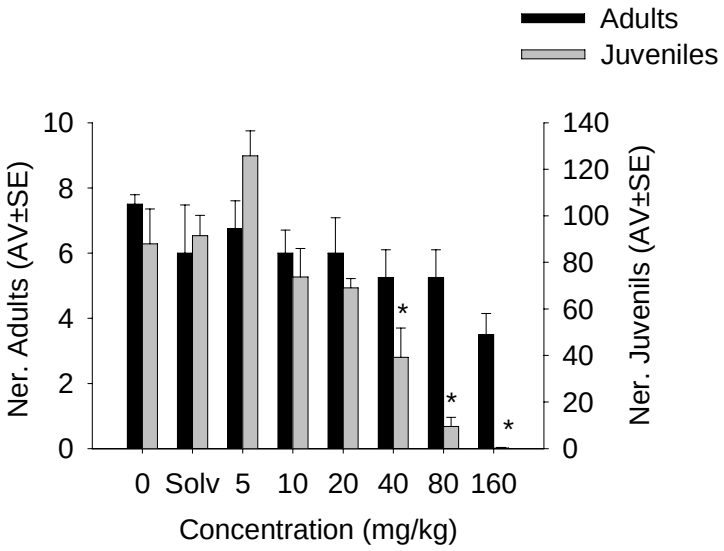

Figure 9 Effects of Phenanthrene in *Enchytraeus albidus* reproduction bioassay, showing number of adults and juveniles. Results are presented as average ± standard error. Asterisks (*) indicate statistically significant differences between control and treatments.

There was no effect of the solvent acetone in either of the measured endpoints. There was no statistically significant differences between control and control solvent (T- test, $p>0.05$). Therefore, control and control solvent were treated as a pool. Adults were not affected, showing only a slight decrease at the highest concentration tested. For juveniles, a dose-response effect could be observed, with a statistical significant decrease (ANOVA Dunnett's method, $p<0.05$) between control and the concentrations of 40, 80 and 160mg/kg. An EC_{50} value for reproduction of 36,8 mg/kg (R^2= 0.7651;

SE= 0.6671) was obtained. All the determined effect levels for survival and reproduction of *E. albidus* can be seen in the following table:

Table 8 Summary of the effect concentrations determined for *Enchytraeus albidus* when exposed to LUFA 2.2 spiked with Phenanthrene.

	EC_{10}	EC_{20}	EC_{50}	NOEC	LOEC
Adults	0.03	0.5	52.1	20	40
95% CI	0-0.5	0-3.2	22.9-217.6		
Juveniles	12.6	19.3	36.8	20	40
95% CI	0.3-21.7	1,9-28.8	20.6-59.6		

Interestingly, there was a significant increase in reproduction when the organisms were exposed to the lowest concentration.

Bioaccumulation test

The overall data on Phenanthrene concentration over time within the 14 days show that the chemical concentration in soil decrease (Figure 10). The decay rate (k_0) derived from (Equation 1) was 0.036 d^{-1} with a standard error of 0.0367. The measured pH values from treated soil ranged between 5.51 and 5.79 in the start and at the end of the uptake phase. Although the pH values at the beginning and at the end of the elimination phase were 5.2 and 5.79, respectively. The pH form the control soil were in the same range.

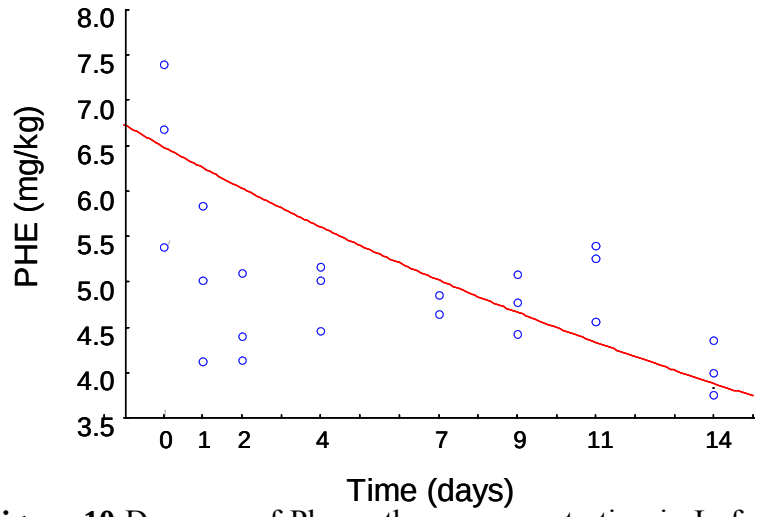

Figure 10 Decrease of Phenanthrene concentration in Lufa 2.2 soil during the 14 days of exposure by GC-MS method. Solid line are based on parameters calculated using Equation 1.

The accumulation and elimination pattern in the present study can be observed in the following figure (Fig. 11).

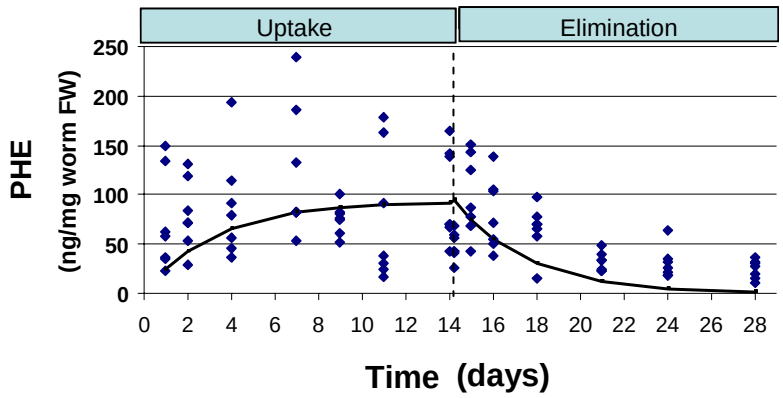

Figure 11 Kinetic behaviour of Phenanthrene in *E. albidus* during the uptake and elimination phase. Solid line results from fitting individual concentration values to models from the equation 2 and 3.

At the end of the assay, in both control and treated worms, the mortality was less than 20%, so the validity criteria were achieved. A weight reduction was observed in the worms during the uptake and elimination phase of the experiment in comparison with test start. Average initial weight was 86.3±11.5mg (Av±SD), decreasing to 74.3±11.2mg (Av±SD) after a 14 days period, corresponding to a 13.8% decrease. Although, at the start of the elimination phase the worms weight was 74.2±8.3mg (Av±SD), decreasing to 47.0±10.6 (Av±SD) corresponding to a 36.6% decrease.

Estimated kinetic parameters are presented in Table 9.

Table 9 Kinetic parameters estimated in the model, where (a) is the assimilation rate value, (k) is the elimination constant rate (with its standard error and p-values associated). and BAF is the bioaccumulation factor.

Parameters	estimate	SE
a	4.379	0.9
k	0.305	0.06
BAF	14,3	

Data points were highly variable.

Discussion and Conclusion

Reproduction

Results for *E. albidus* bioassay revealed a high toxicity of Phenanthrene to this organism. Both survival and reproduction were affected at lower concentrations. The EC_{50} value for reproduction determined was 36.4mg/kg and the LC_{50} was 52.1mg/kg. Interestingly it was observed a slight enhancement of reproduction in the lowest tested concentration. Studies performed in Oligochaete species such as, *Enchytraeus crypticus* (Sverdrup et al., 2002) and *Eisenia fetida* (Contreras-Ramos et al., 2006) reported EC50s of 40mg/kg and 100mg/kg respectively, therefore in the same order of magnitude. Also in a study with collembolans, *Folsomia fimetaria* (Sverdrup et al., 2001) a very similar EC_{50} value for reproduction of 30mg/kg was reported.

Bioaccumulation

Kinetics studies should be performed under nontoxic levels, at dosages in which toxic damage occurs on survival, growth or reproduction. Therefore in the present experiment a concentration of PHE of 8mg/kg was tested to guarantee a healthy condition in the organisms. The results show that *E. albidus* is able to accumulate PHE and that the test design, was appropriate to assess the bioaccumulation in these worms. The equilibrium state was achieved and it was possible to fit the data to the model and estimate the different parameters.

For *Eisenia andrei* (Jager et al., 2000) experiments with PHE yield an uptake constant of 1.4kg/kg/d and an elimination rate of $0.16d^{-1}$, and a BAF of 7.3. *E. albidus* had a much higher uptake rate.

Accumulation by earthworms in soil have two major uptake routes: soil particles and the intersticial water (Krauss et al., 2000). In a general way, studies with less hydrophobic contaminants (log k_{ow}<5), show evidence that accumulation is mainly via interstitial water; whereas studies with more hydrophobic chemical (log k_{ow}>5-6) show evidence of significant uptake from soil particles (Belfroid et al., 1996). Sorption to soil is one of the controlling factors of its bioavailability (Belfroid and Sijm, 1998). PAHs tend to adsorb to soil considerably, thus decreasing the concentration in interstitial water considerably (Belfroid et al., 1996). Sorption process is governed by physico-chemicals properties of the chemical (water solubility and hydrophobicity) and by characteristics of the soil (e.g. organic matter content, clay content) (Belfroid et al., 1996). The association with soil-chemical is larger for chemicals with low water solubility (high hidrophobicity) and for soils with a high organic carbon and clay content (Rodgers et al., 1987). In the present study, the tested soil has an organic matter content of 4,4% a

low clay content and given the fact that the $\log_{kow}$ is 4.6, it is likely that PHE is in both fractions: soil and interstitial water, and therefore more bioavailable to the organism through the aqueous phase. Nevertheless it is known that these organisms also ingest soil particles and can be affected by the soil itself.

The comparison of our data with other chemicals data with similar log k_{ow} is of major importance. The accumulation of chlorobenzenes in earthworms (*E. andrei*) kept in artificial OECD soil was studied (Belfroid et al., 1993). Three PAHs where tested: 1, 2, 3, 4-tetrachlorobenzene, with a logk_{ow} similar to Phenanthrene, showed a biphasic elimination phase, with an initial fast elimination in 2 days followed by a slower decrease. For the other PAHs, pentachlorobenzene and hexacholorobenzene, the initial phase lasted 6 and 10 days, respectively.
n the present study, is not possible to define exactly the elimination behaviour of PHE due to high data variability.

The observed decrease of Phenanthrene in the soil in the present study could be due to experimental factors. Additionally, as earthworms improve aeration and the conditions for microbial activity therefore increase the biodegradation of PAHs. (Eijsackers et al., 2001)
The FF method was efficiently measuring the PHE content in the organisms. This method is commonly used for the assessment of PAHs concentration, e.g. in certain organs of fish, such as bile, vesicular, liver, cytosol, etc (Lin et al., 1996; Aas et al., 1998). GCMS for soil analysis proved to be an apropriate method.

Overall, this study showed that the phenanthrene causes harmful effects to *Enchytraeus albidus*. This test species accumulates PHE, and it was possible estimate the kinetic parameters.

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